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ORIGINAL ARTICLE

Identification of a novel homolog for a calmodulin-binding protein that is upregulated in alloplasmic wheat showing pistillody

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Abstract Intracellular signaling pathways between the mitochondria and the nucleus are important in both normal and abnormal development in plants. The homeotic transformation of stamens into pistil-like structures (a phenomenon termed pistillody) in cytoplasmic substitution (alloplasmic) lines of bread wheat (*Triticum aestivum*) has been suggested to be induced by mitochondrial retrograde signaling, one of the forms of intracellular communication. We showed previously that the mitochondrial gene *orf260* could alter the expression of nuclear class B MADS-box genes to induce pistillody. To elucidate the interactions between *orf260* and nuclear homeotic genes, we performed a microarray analysis to compare gene expression patterns in the young spikes of a pistillody line and a normal line. We identified five genes that showed higher expression levels in the pistillody line. Quantitative expression analysis using real-time PCR indicated that among these five genes, *Wheat Calmodulin-Binding Protein 1* (*WCBP1*) was significantly upregulated in young spikes of the pistillody line. The amino acid sequence of WCBP1 was predicted from the full-length cDNA sequence and found to encode a novel plant calmodulin-binding protein. RT-PCR analysis indicated that *WCBP1* was preferentially expressed in young spikes at an early stage and decreased during spike maturation, indicating that it was associated with spikelet/floret development. Furthermore, in situ hybridization analysis suggested that *WCBP1* was highly expressed in the pistil-like stamens at early to late developmental stages. These results indicate that *WCBP1* plays a role in formation and development of pistil-like stamens induced by mitochondrial retrograde signaling.

Keywords *Aegilops* · Cytoplasm · Homeosis · Microarray · Mitochondrial retrograde signaling · *Triticum*

Abbreviations

CaM	Calmodulin
CMS	Cytoplasmic male sterility
Ctg	Contig
CS	Chinese Spring
MRS	Mitochondrial retrograde signaling
N26	Norin 26

Introduction

The intracellular signaling between mitochondria and the nucleus is important for maintaining the normal physiological state in the cell. This communication occurs

The nucleotide sequence for *WCBP1* cDNA reported in this paper has been submitted to the DDBJ/EMBL/GenBank database under the accession number AB716956. The wheat oligomicroarray with 37,826 probes is registered as GPL9805 in GEO at NCBI. A complete set of microarray data from this study was deposited to the GEO repository under the accession number GSM920888-920891.

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through a pathway termed mitochondrial retrograde signaling (abbreviated here as MRS) (Butow and Avadhani 2004; Liu and Butow 2006). In yeast, four positive regulatory factors (Rtg1p, Rtg2p, Rtg3p and Grr1p) and four negative regulatory factors (Mks1p, Lst8p, Bmh1p and Bmh2p) have been identified to date as being of importance for MRS. These proteins constitute a feedback control for monitoring mitochondrial activity, and for up-regulating nuclear target genes. In mammalian cells, MRS occurs through an increase in cytosolic Ca^{2+}. The divalent Ca^{2+} cation is a second messenger in endogenous and exogenous signaling pathways for inducing cellular responses, and mitochondrial Ca^{2+} handling is involved in MRS (Szabadkai and Duchen 2008). Furthermore, TOR (target of rapamycin) signaling has also been shown to be linked to MRS in yeast and animals (Butow and Avadhani 2004). TOR is a Ser/Thr kinase that is highly conserved in eukaryotes. Compared with MRS in yeast and animals, relatively little information on plant MRS is available at present.

To investigate the interaction of cytoplasmic and nuclear genomes with respect to plant phenotype, cytoplasmic substitution (alloplasmic) lines of bread wheat (*Triticum aestivum*) that carry the cytoplasm of the wild relative *Aegilops crassa* were produced by recurrent backcrossing (Tsunewaki et al. 1996, 2002). In one such alloplasmic line in which *Ae. crassa* cytoplasm was introduced into the wheat cultivar (cv.) Norin 26 (N26), the plants showed homeotic transformation of stamens into pistil-like structures (pistillody) under long day conditions (>15-h light period) and were therefore male sterile (Murai and Tsunewaki 1993). This phenomenon was named photoperiod-sensitive cytoplasmic male sterility (PCMS) and has been shown to have practical applications in hybrid wheat breeding (Murai et al. 2008). In contrast to cv. N26, the cv. Chinese Spring (CS) does not show pistillody when *Ae. crassa* cytoplasm is introduced because of the action of a single dominant gene (designated *Rfd1*) located on the long arm of chromosome 7B (Murai and Tsunewaki 1994). To investigate the function of the *Rfd1* gene, an alloplasmic line of CS with ditelosomy of chromosome 7BS (i.e., lacking the long arm of chromosome 7B) and with *Ae. crassa* cytoplasm was produced. This line is named (cr)-CSdt7BS and, in the absence of *Rfd1*, exhibits pistillody irrespective of photoperiod. By contrast, cv. CS plants with ditelosomy of 7BS but with a normal cytoplasm (CSdt7BS) form normal stamens (Murai et al. 2002). These results indicate that pistillody is induced by factor(s) in the *Ae. crassa* cytoplasm, presumably from the mitochondrial genome, and that the nuclear *Rfd1* gene prevents the deleterious effects of the cytoplasm. PCMS in alloplasmic lines of cv. N26 might involve an *Rf* gene that functions under short day conditions. One candidate for a cytoplasmic factor causing pistillody in alloplasmic wheat is the *Ae. crassa* mitochondrial gene *orf260*, which is predicted to encode a membrane protein (Zhu et al. 2008). Another possibility is retrograde signaling via a protein kinase encoded by the wheat pistillody-related protein kinase 1 (*WPPK1*) (Saraike et al. 2007).

Studies in flowering plants, particularly *Arabidopsis*, have shown that five classes of homeotic genes (termed A, B, C, D and E) define floral organ identity (Zahn et al. 2006). The class B, C and E genes specify stamens in the third floral whorl, and class C and E genes specify carpels in the inner fourth whorl. Various genes from these classes have been identified in *Arabidopsis*: the class B genes *APETALA 3* (*AP3*) and *PISTILLATA* (*PI*); the class C gene *AGAMOUS* (*AG*); and the class E genes, *SEPALLATA 1* (*SEP1*), *SEP2*, *SEP3* and *SEP4*. All of these homeotic genes encode MADS-box transcription factors (Riechmann and Meyerowitz 1997). Loss-of-function of class B MADS-box genes results in the homeotic transformation of stamens into carpel- or pistil-like structures in *Arabidopsis* (Jack et al. 1992; Goto and Meyerowitz 1994) and *Antirrhinum* (Sommer et al. 1990; Tröbner et al. 1992). Similarly, in monocots, the class B gene mutant *silky1* in maize and *superwoman1* in rice show male sterility due to homeotic conversion of stamens into carpels (Ambrose et al. 2000; Nagasawa et al. 2003). From these findings, it is likely that wheat class B MADS-box genes might be associated with pistillody induction in alloplasmic wheat. Previous studies identified a wheat *AP3* ortholog, *WAP3* (*wheat APETALA3*) (Murai et al. 1998), and two wheat *PI* orthologs, *WPI-1* (*wheat PISTILLATA-1*) and *WPI-2* (Hama et al. 2004), associated with pistillody. *WAP3* and *WPI* are wheat class B genes and are down-regulated in the primordia of pistil-like stamens during pistillody. These results clearly indicate that the induction of pistillody is associated with alterations in the expression patterns of class B MADS-box genes in alloplasmic wheat (Hama et al. 2004).

The present study was initiated to identify the nuclear gene(s) regulated by MRS between the pistillody-related mitochondrial gene *orf260* and nuclear class B MADS-box genes. To achieve this aim, we performed a microarray analysis using a custom wheat oligomicroarray (Kawaura et al. 2006, 2008; Tang et al. 2011). This analysis enabled us to identify a novel gene for a calmodulin (CaM)-binding protein (*Wheat Calmodulin-Binding Protein 1*, *WCBP1*) that was upregulated in the pistillody line. Expression and in situ hybridization experiments indicated that upregulated expression of *WCBP1* played a role in the formation and development of pistil-like stamens in the alloplasmic line. The fact that a CaM-binding protein is associated with pistillody induction suggests that MRS in plants involves a mechanism related to Ca^{2+} signaling pathways similar to that described for yeast and mammalian cells.

Materials and methods

Plant materials

Bread wheat (*Triticum aestivum*) cv. Chinese Spring (CS) (obtained from the National Bioresource Project—Wheat (NBRP–KOMUGI), http://www.shigen.nig.ac.jp/wheat/komugi/top/top.jsp) was used for identification and expression analysis of *WCBP1* (*Wheat Calmodulin-Binding Protein 1*). A cytoplasmic substitution line of CS carrying *Aegilops crassa* cytoplasm and which has ditelosomy of chromosome 7BS (line name (cr)-CSdt7BS) was obtained from the Department of Bioscience, Fukui Prefectural University, Japan; this line lacks the chromosome 7B long arms (7BL). The (cr)-CSdt7BS substitution line was used for the microarray analysis, expression analysis and in situ hybridization analysis. The line exhibits pistillody due to the absence of the *Rfd1* gene located on 7BL, which normally prevents the effect of the *Ae. crassa* cytoplasm on stamen formation (Murai et al. 2002). A euplasmic CS ditelosomic 7BS line with normal cytoplasm (line name CSdt7BS) was also obtained from the Department of Bioscience, Fukui Prefectural University, Japan; this line shows a normal phenotype and was used as the control for the various analyses.

Microarray analysis

Total RNA was isolated using ISOGEN reagent (Nippon Gene) from young spikes at the floret differentiation stages (3–10 mm in length) of plants from the pistillody line (cr)-CSdt7BS and the control line CSdt7BS. Three to five young spikes were obtained from each of five plants. Total RNA was isolated from the combined samples of each line. The RNAs were then labeled with Cy3 using a Low RNA Input Linear Amp kit (Agilent Technologies) according to the manufacturer's instructions. Aliquots of Cy3-labeled cRNAs were used for hybridization to the custom wheat 38k oligomicroarray (Agilent Technologies). After hybridization, the microarray slides were scanned using a G2505B scanner (Agilent Technologies) and data analysis was performed using Feature Extraction software (version 9.5; Agilent Technologies) with the default settings. Subsequently, the data were analyzed using GeneSpring GX7.3 software (Agilent Technologies). False positives were controlled by measuring the false discovery rate (Benjamini and Hochberg 1995). We performed the hybridization assay with two technical replications. This Agilent Wheat oligomicroarray with 37,826 probes is registered as GPL9805 in the Gene Expression Omnibus (GEO) at the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/geo/). All expression tag contig sequences used in the design of probes have been published online (KOMUGI, http://www.shigen.nig.ac.jp/wheat/komugi/array/probe/download.jsp). A complete set of microarray data from this study was deposited in the GEO repository under accession number GSM920888-920891.

Real-time PCR analysis

For the analysis of expression of candidate pistillody-related genes identified by the microarray analysis, total RNAs were isolated from young spikes at the floret differentiation stage (3–10 mm in length) of plants from the pistillody line (cr)-CSdt7BS and the control line CSdt7BS. Spikes were obtained from five individual plants, and total RNAs were isolated from the combined sample of each using ISOGEN (Nippon-gene). DNase-digested total RNA (4.5 μg) was reverse-transcribed with oligo-dT primer and first-strand cDNA was obtained using a first-strand synthesis kit for RT-PCR (GE Healthcare Biosciences). PCR primers for RT-PCR were designed from the sequences of the candidate pistillody-related genes, including *WCBP1*, as indicated in Table 1. As a control, a wheat ubiquitin gene (*Ubi-1*) sequence was amplified using the primers Ubi-1L (5′-GCATGCAGATATTTGTGAA-3′) and Ubi-1R (5′-GGAGCTTACTGGCCAC-3′) (Murai et al. 2002). Quantitative real-time PCR experiments were performed using a LightCycler 2.0 thermal cycler (Roche Diagnostics GmbH), and in all cases quantities were estimated by SYBR Green I fluorescence using the *Ubi-1* gene as the endogenous control.

Table 1 Pistillody-related genes (contigs) identified by a microarray analysis, and the specific primer sequences used for amplification

Contig no.	Annotation	PCR primer sequence (5′–3′)
Ctg 6837	RelA/SpoT protein	TTGAAGGGGAAACAAAGGTG
		TGGCGATAGCATACTGCTTG
Ctg 9545	F-box domain containing protein	GGTGGCAGAGATTCAGCTTC
		GTCCAGGATGCTCGTTTAGC
Ctg 4015	Calmodulin-binding protein	CATATCAGGAGGGTGCCACT
		TGACCGTCACTGCAGAAGTC
Ctg 10001	Protein kinase domain containing protein	CAAGCATGAAAACCTCGTCA
		TGTTGCTCGCTTTTATGTCG
Ctg 2065	CRAL/TRIO domain containing protein	GGAAGCACGCTTCTCATTTC
		GTCCTCAGGCCTGCTAACTG

RT-PCR analysis

Spikes at various developmental stages (<3 mm, 3–10 mm, 10–15 mm and 15–25 mm in length; flag leaf unfolding and booting stages) were sampled from normal CS plants and total RNAs were isolated using ISOGEN (Nippon-gene). Three or more individual plants were sampled at each developmental stage, and total RNAs were isolated from the combined sample. For isolation of RNA from young spikes less than 10 mm in length, more than ten plants were required to obtain sufficient tissue. Total RNAs were also isolated from leaves, stems and roots of seedlings at vegetative stage (the 3-leaf stage) and of plants at the reproductive stage (floret differentiation stage); in each case three individuals were sampled. RT-PCR analysis was performed in the exponential range of amplification. PCR fragments were separated on a 1.5 % agarose gel, stained with ethidium bromide and photographed. Each PCR assay was performed three times with different numbers of amplification cycles. Invariably, band intensities were lowest in the PCRs with the smallest number of cycles, indicating that the amplification must still have been in the exponential phase. The primer sets used for the RT-PCR analysis were same as those for the real-time PCR analysis.

In silico expression profiling analysis

Because the sequenced cDNA were more or less changed in their libraries, the expression pattern of each gene in certain tissues after certain treatments could be monitored by counting the constituents in the EST database (Kawaura et al. 2005). From the libraries deposited in the EST database of KOMUGI-Wheat Genetic Resources Database (http://www/shigen.nig.ac.jp/wheat/komugi/), we selected three to perform an expression profiling analysis for abiotic stress (library names SCTAL, VHS, V4816) and three others to perform an expression profiling analysis for biotic stress (library names CHUL, XF, XP. SCTAL). VHS and V4816 are cDNA libraries from seedlings treated with aluminum, cold and heat shock, respectively. CHUL, XP and XP are cDNA libraries from seedlings infected with powdery mildew, leaf rust and blast, respectively. Detailed information on the libraries is available from the KOMUGI database.

In situ hybridization analysis

In situ hybridization analysis was performed using the method described previously (Kinjo et al. 2012; Shitsukawa et al. 2009). Young spikes at the spikelet primordial to floral organ developing stages from plants of the (cr)-CSdt7BS and CSdt7BS lines were fixed with FAA solution (3.7 % p-formaldehyde, 5 % acetic acid) at 4 °C overnight, dehydrated, and then embedded in Paraplast resin (Oxford Labware). The embedded tissues were used to cut 8-μm sections, which were dried overnight onto Vectabond (Vector Laboratories)-coated slides. Tissue sections were deparaffinized with xylene and rehydrated through a descending ethanol series, treated with proteinase K and triethanolamine, then dehydrated through an ascending ethanol series. DIG-labeled RNA probes were synthesized from plasmids containing a 3′ region of *WCBP1* cDNA (about 1 kb in length: *Xho* I site to 3′ end) by in vitro transcription using a DIG RNA labeling kit (Roche Diagnostics). Hybridization was performed overnight at 52 °C. After hybridization, sections were washed and treated with RNase. The slides were soaked in 0.5 % blocking reagent (Roche Diagnostics) and anti-digoxigenin alkaline phosphatase conjugate containing 0.1 % BSA. After the slides were washed, transcripts were detected with NBT/BCIP (Roche Diagnostics).

Sequence analysis

Multiple amino acid sequence alignment of plant calmodulin (CaM)-binding proteins and the WCBP1 protein identified in this study was carried out using the computer program clustalw2 (Larkin et al. 2007) with matrix Blosum (gap open penalty, 10; gap extension penalty, 0.2; gap distance, 5), and a phylogenetic tree was constructed using the neighbor-joining method (Saitou and Nei 1987). The programs used here were provided by EMBL-EBI (European Bioinformatics Institute) databases (http://www.ebi.ac.uk/Tools/msa/clustalw2/). The amino acid sequences of CaM-binding proteins were obtained from the NCBI database, and the accession numbers and references for each are given in Table 2. Domain analysis of WCBP1 was performed using the computer program ProDom (the Protein Domain Database, http://prodes.toulouse.inra.fr/prodom/doc/prodom.html).

Statistical analysis

For real-time PCR analysis, *t* tests were used applied to compare the gene expression levels between CSdt7BS (normal) line and (cr)-CSdt7BS (pistillody) line.

Results

Microarray analysis for identification of pistillody-related genes in an alloplasmic wheat line

Plants of the CSdt7BS line, which have a normal CS cytoplasm and ditelosomy of 7BS (and therefore lack

Table 2 Accession numbers of proteins (genes) used for the phylogenetic analysis

Gene name	Accession no.	Species	References
WCBP1	AB716956	*Triticum aestivum*	This study
TaHSP101	AAD22629	*Triticum aestivum*	Campbell et al. (2001)
NtCBP60	AAB37246	*Nicotiana tabacum*	Unpublished
NtGAD1	AAC24195	*Nicotiana tabacum*	Unpublished
NtKinesin-like	AAC49393	*Nicotiana tabacum*	Wang et al. (1996)
NtCNGC	AAF33670	*Nicotiana tabacum*	Arazi et al. (1999)
NtCBHP	AAB34987	*Nicotiana tabacum*	Lu et al. (1995)
NtEr1	AAG39222	*Nicotiana tabacum*	Yang and Poovaiah (2000)
NtCaMK3	AAL30820	*Nicotiana tabacum*	Hua et al. (2003)
NtCaPK1	AAN71903	*Nicotiana tabacum*	Ma et al. (2004)
ZmCBP	NP_001150045.1	*Zea mays*	Alexandrov et al. (2009)
ZmECA	AAF73985	*Zea mays*	Subbaiah and Sachs (2000)
OsGAD	BAB32868	*Oryza sativa*	Akama et al. (2001)
OsCBP	AAP85535	*Oryza sativa*	Unpublished
OsCNGC	AAK16188	*Oryza sativa*	Unpublished
OsCaMBTF	AAQ07306	*Oryza sativa*	Choi et al. (2005)
GmKinesin-like	XP_003547877.1	*Glycine max*	Unpublished
GmCaMBTA2	XP_003547081.1	*Glycine max*	Unpublished
GmACA	AAG28435	*Glycine max*	Chung et al. (2000)
HvCNGC	CAA05637	*Hordeum vulgare*	Schuurink et al. (1998)
LeER66	AAD46410	*Lycoperisicon esculentum*	Zegzouti et al. (1999)
LeCaMBSRiL	AEX31181	*Lycoperisicon esculentum*	Yang et al. (2012)

Rfd1), form normal stamens. By contrast, plants in the alloplasmic (cr)-CSdt7BS line, which have *Ae. crassa* cytoplasm, exhibit pistillody in all florets (Fig. 1). We performed a microarray analysis to screen for genes with altered transcript levels between the pistillody line and the normal line. A custom wheat 38k oligomicroarray was used to compare overall gene expression patterns in CSdt7BS and (cr)-CSdt7BS plants. This wheat microarray can be

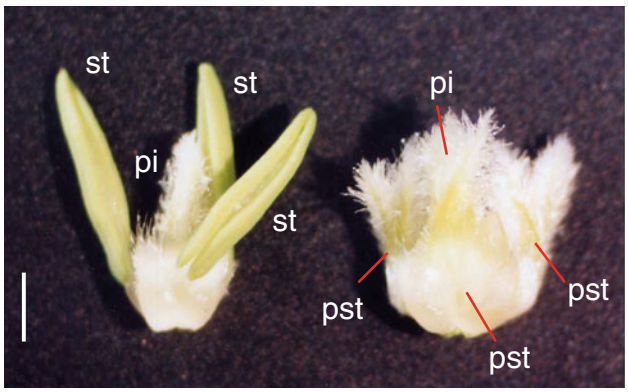


Fig. 1 Floral organs in plants from the normal CSdt7BS line and the pistillody (cr)-CSdt7BS line. The lemma and palea have been detached from the floret. *pi* pistil, *st* stamen, *pst* pistilloid stamen; *bar* 2 mm

used to identify genes that show specific increases in expression levels in (cr)-CSdt7BS. The analysis found five annotated cDNA contigs (Ctg6837, Ctg9545, Ctg4015, Ctg10001 and Ctg2065) with increased transcript levels (more than 30-fold) in (cr)-CSdt7BS plants compared to the control CSdt7BS plants. Annotation was performed by the KOMUGI-Wheat Genetic Resources Database (http://www/shigen.nig.ac.jp/wheat/komugi/). The identified annotations of the Ctgs are given in Table 1. The E-value of microarray analysis in Ctg6837, Ctg9545, Ctg4015, Ctg10001 and Ctg2065 are 1.13E−142, 3.99E−16, 2.45E−65, 3.62E−96 and 4.88E−47, respectively. The detailed data from the microarray analysis are available through GEO in NCBI, as indicated in the "Materials and methods".

Using specific primers for each of the Ctgs, real-time PCR analyses were performed with cDNAs of young spikes to confirm the specificity of the expression patterns (Fig. 2). The real-time PCR analysis indicated that two of the five genes, Ctg4015 and Ctg10001, had a significantly higher level of expression in the (cr)-CSdt7BS (pistillody) plants compared to the control CSdt7BS (normal) plants. Ctg4015 was found to have sequence similarity to calmodulin (CaM)-binding proteins, and Ctg10001 appeared to encode a protein with a protein kinase domain (Table 1). This suggests a relationship between expression of Ctg4015 and Ctg10001 and the induction of pistillody in the alloplasmic line. Real-time PCR analysis also indicated that there was no significant difference in the expression levels of Ctg6837, Ctg9545 and Ctg2065 between the two lines. This result indicates that the microarray analysis is not suitable for identification of difference in expression level of genes with low expression levels, such as Ctg6837, Ctg9545 and Ctg2065.

Ctg4015 is preferentially expressed in young spikes

We investigated the expression profile of Ctg4015 and Ctg10001 during spike formation using RT-PCR analysis of normal CS plants at the spikelet differentiation stage (<3 mm in length), the floret differentiation stage (3–10 mm), the floral organ early developing stage (10–15 mm), the floral organ late developing stage (15–25 mm), the flag leaf unfolding stage (floral organ maturing stage), and booting stage (meiosis stage) (Fig. 3). Ctg10001 showed almost constant expression levels throughout spike development. By contrast, Ctg4015 showed variation in expression with the highest level in spikes less than 10 mm in length, coinciding with the spikelet differentiation to floret differentiation stages; expression decreased during spike maturation. This suggests that Ctg4015 plays a specific role in young spikes at the early stages during spike development.

Ctg4015 encodes a predicted novel calmodulin-binding protein

We performed a BLAST search of the wheat EST database to identify the cDNA clone corresponding to Ctg4015 (Ogihara et al. 2004; Kawaura et al. 2009; provided in KOMUGI-Wheat Genetic Resources Database, http://www/shigen.nig.ac.jp/wheat/komugi/). Ctg4015 was found to be part of the full-length cDNA clone whrd12j19 that encodes a 555 amino acid polypeptide and shows homology to calmodulin (CaM)-binding proteins. Therefore, we renamed Ctg4015 as *Wheat CaM-Binding Protein 1* (*WCBP1*).

To examine the relationship between WCBP1 and other plant CaM-binding proteins, a phylogenetic tree was constructed using the predicted amino acid sequences (Fig. 4). The list of plant CaM-binding proteins used for this analysis is given in Table 2. The phylogenetic tree indicated that the plant CaM-binding proteins could be classified into eight groups: CaM-binding protein 60 (CBP60), glutamate decarboxylase (GAD), kinesin-like CaM-binding protein (KL), cyclic nucleotide-gated CaM-binding ion channel (CNGC), anther ethylene-upregulated protein ER (ER), CaM-binding transcription factor (TF), plasma membrane Ca^{2+}-ATPase (CaATP), and CaM-dependent protein kinase (PK) families. This classification is consistent with that described by Reddy et al. (2002). CaM-binding heat shock proteins from wheat (TaHSP101) and tobacco (NtCBHP) were not in the same clusters. WCBP1 was in a cluster with CBP60 proteins of tobacco (NtCBP60) and maize (ZmCBP), indicating its similarity to the CBP60 family. However, WCBP1 does not have the consensus amino acids region of a CaM-binding domain, as is present

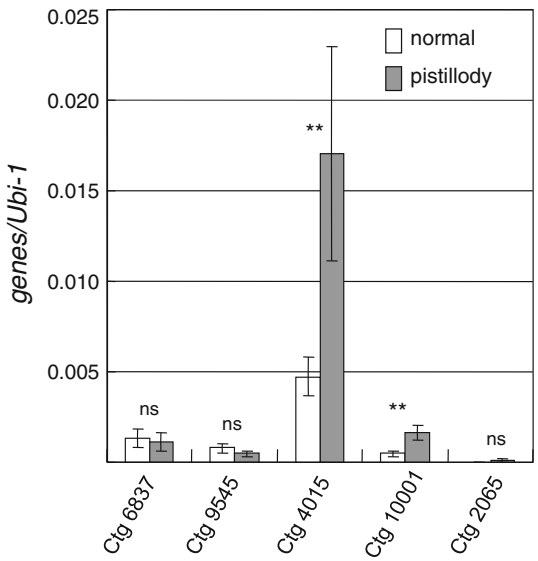


Fig. 2 Expression analysis of five contigs (Ctgs) by real-time PCR. Total RNA was isolated from young spikes at the floret differentiation stage of the pistillody line and normal line. Expression levels were normalized against the *Ubiquitin* (*Ubi-1*) gene. Error bars represent standard errors. Statistical analysis was performed by *t* tests to assess differences in expression levels between normal and pistillody lines. *ns* not significant, **significantly different at $P = 0.01$

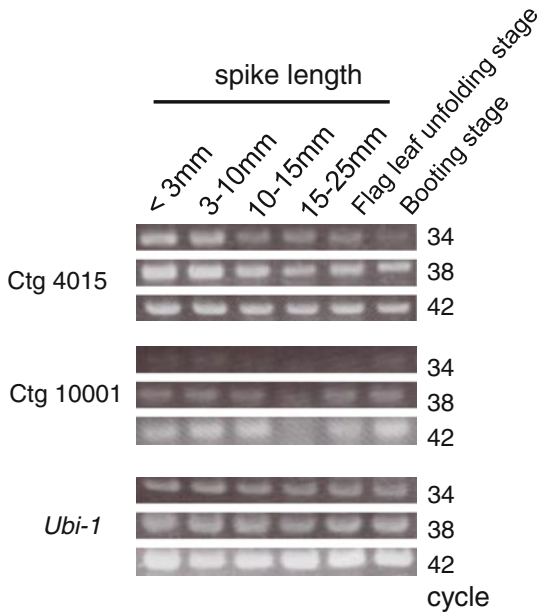


Fig. 3 Expression analysis of contigs (Ctgs) 4015 and 10001 in a normal line (cv. Chinese Spring) by RT-PCR. The *Ubiquitin* (*Ubi-1*) gene was used as the control, showing that the same amount of cDNA was used in PCR for samples. Each PCR assay was performed three times with different numbers of amplification cycles. Total RNAs were isolated from young spikes less than 3 mm in length, corresponding to the stage of spikelet differentiation, and from young spikes (3–10 mm and 10–15 mm in length) corresponding to the floret differentiation to floral organ developing stages. Total RNAs were also isolated spikes from the plants at the flag leaf unfolding stage (floral organ maturing) and booting stage (meiosis)

in CBP60 family proteins such as NtCBP60 and ZmCBP (Fig. 5). Analysis of WCBP1 using the ProDom program indicated that the protein does have three other domains that are specific to CaM-binding proteins. These results confirm that WCBP1 is a novel type of plant CaM-binding protein.

Expression profile of *WCBAP1*

The expression level of *WCBP1* in various organs of seedlings at the vegetative stage (the 3-leaf stage) and in plants at the reproductive stage (floret differentiation stage) was investigated by RT-PCR analysis (Fig. 6). *WCBP1* was expressed all tissues (leaves, stems, and roots) at both vegetative and reproductive stages, although the expression level varied. This suggests that *WCBP1* functions in these different tissues in a range of developmental stages.

To examine the effect of abiotic or biotic stress on *WCBP1* expression, we performed an in silico expression profiling analysis using a large scale EST database from the KOMUGI-Wheat Genetic Resources Database (http://www/shigen.nig.ac.jp/wheat/komugi/). Expression levels were estimated as the ratio of the *WCBP1* sequence among the total EST clones (counts per million) (Fig. 7). In silico expression profiling analysis suggests that *WCBP1* expression responded to aluminum treatment but not to heat shock or cold treatments. Furthermore, *WCBP1* expression was also altered by biotic stress: upregulation by infection of powdery mildew, but not by leaf rust or blast. These results suggest that *WCBP1* functions in specific stress signaling pathways.

In situ hybridization analysis of *WCBP1* in developing young spikes

Next, we carried out an in situ hybridization analysis to determine the distribution of *WCBP1* in young spikes from control CSdt7BS and pistillody (cr)-CSdt7BS plants at the floret differentiation stage and at the floral organ developing stage in which stamen and pistil primordia are differentiated (Fig. 8). Each wheat floret contains a pistil, stamens and lodicules, and is enclosed by leaf-like structures, the lemma and palea (Murai et al. 2002). Lodicules are modified petals and the lemma and palea are probably modified sepals. In a longitudinal section of a young floret from a CSdt7BS plant, we detected *WCBP1* mRNA in the apical region of the spikelet primordia (Fig. 8a). Expression was then detected in the primordia of both the pistil and stamens at the floret differentiation stage (Fig. 8c). At the late floret differentiation stage, *WCBP1* mRNA mainly

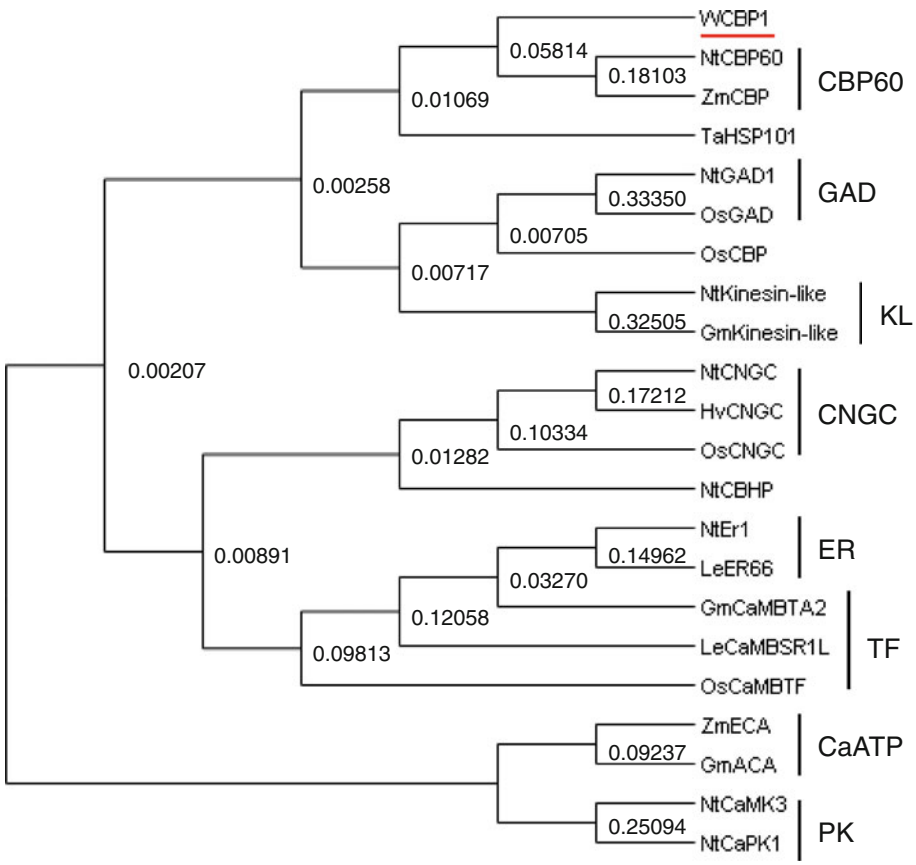


Fig. 4 Phylogenetic tree constructed using the amino acid sequences of the calmodulin (CaM)-binding protein family from various plant species together with WCBP1. The phylogenetic tree was constructed by the neighbor-joining method using predicted amino acid sequences. The numbers at the nodes show bootstrap values. Amino acid sequences of CaM-binding protein genes were obtained from the NCBI database, and the accession numbers and references of each gene are given in Table 2. The CaM-binding proteins formed eight clusters: CaM-binding protein 60 (CBP60), glutamate decarboxylase (GAD), kinesin-like CaM-binding protein (KL), cyclic nucleotide-gated CaM-binding ion channel (CNGC), anther ethylene-upregulated protein ER (ER), CaM-binding transcription factor (TF), plasma membrane Ca^{2+}-ATPase (CaATP), and CaM-dependent protein kinase (PK) families

```
NtCBP60   ------------------------------------------------MEETCS------ 6
ZmCBP     -------MKEKRGLEDAGGGAGDGRPDAKRSRPPALASVIVEALKMDSLQRLCSSLEPIL 53
WCBP1     MRRTVLVVLFLLRMSDGITVTESISQIGRMVQRKMEGIQKVQALMMRKLENLEEKVESMS 60
                                                             ::.  .

NtCBP60   -------FSSGLGYL-------------------------------YPLFTGAKVEGEHG 28
ZmCBP     RRVVSEEVERALGRLGPATIGGRSSPKRIEGPDGRALQLQFRTRLSLPLFTGGKVEGDQG 113
WCBP1     HEVKKLARLHSNRHADKYPRSEPYQEGATLSEPNANIRLRFLDGLRTPIYTDKIITSQSN 120
                     .                                        *::*.  : .: .

NtCBP60   AAIHVVLIDADTGHLVTTGAESCIKLDVVVLEGDFNTEDDEGWTQEEFDSHVVKEREGKR 88
ZmCBP     AAIHVVLLDAGTGGVVSSGPESSAKLDIVVLEGDFNNEDEEGWTGEEFDSHVVKEREGKR 173
WCBP1     AAIRVGVFDGEK--MINEGPLSKAKVEILVLRGDFCSDGQESWTEEEFNSHIAQGRHRQG 178
          ***:* ::*. .  ::. *. *  *::::**.*** .:.:*.** ***:**:.: *. :

NtCBP60   PLLTGELQVTLKGGVGTLGDLTFTDNSSWIRSRKFRLGMKVASG-YCEGVRIREAKTEAF 147
ZmCBP     PILTGDLQVTLKEGVGTIGELTFTDNSSWIRSRKFRLGLKLASG-FCEGVRIREAKTEAF 232
WCBP1     SVLGGDCSAWLNNGEASLGKIRFREGSSRTPSRKFIVGGRVCMNRKIGGIRVQEAVMEPV 238
          .:* *: .. *: * .::*.: * :.**   **** :* ::. .    *:*::**  *..

NtCBP60   TVEDHRGELYKKHYPPALNDDVWRLEKIGKDGSFHKRLNKAAIFTVEDFLRLVVRDPQKL 207
ZmCBP     MVKDHRGELYKKHYPPTLKDEVWRLEKIGKDGSFHKRLNKSGISTVEDFLRLVIRDPQKL 292
WCBP1     TVLDRRNEANEKRHPPRLDDEVYRLEEISRDGIYHRRLKNVHIFTVEDFLKALNKDADVL 298
           * *:*.*  :*::** *.*:*:***:*.:** :*:**::  * ******: : :*.: *

NtCBP60   RNILGSG-MSNKMWDALIEHAKTCVLSG--KLYVYYSDDSRNVGVVFNNIYELNGLIAGE 264
ZmCBP     RSILGSG-MSNKMWDALVEHAKTCVLSG--KYYIYYSDESRSIGAIFNNIYAFCGLISGE 349
WCBP1     REMVLEIKKRSNAWERMVKHARECCLADRPELKAYHSVEG-NVVIFFNCVHDLVGAIFYG 357
          *.:: .    .: *: :::**: * *:.  :   *:* :. .:  .** :: : * *

NtCBP60   QYYSADSLSDSQKVYVDSLVKKAYDNWNQVVEYDGKSFLNIKQNQKAGSSRNELPVGPVD 324
ZmCBP     QFYSSESLDDSQKLFADALVKKAYDNWMYVIEYDGKGLLNLKPKKKAASTGQAETHASAG 409
WCBP1     VYVSRDNFDPAKKAQAYELKECARG-QLDILPFD--YVMNGNLPEQVPSSTHSTLDAAFI 414
           : * :.:. ::*  .  * : * .    :: :*   .:* :  ::. *: :    ..

NtCBP60   YPNNMVNQLPQSRLPVSVQSEQSSMDPNLLIGGSGYNDSIVARMPNQSQMMNSSSRSQFE 384
ZmCBP     GSTSYQQHLSSTSMPGSSPS-----------GGTDSVGYGVDQSATHLSQLQSSSTN--L 456
WCBP1     LGPDVALQPNENQLQDRINN--------------------ITEPSHQNEYIHGDQST--- 451
             .   :  .. :     .                     : . . : . ::... .

NtCBP60   STPFAPQQQITNTHQLQSTSYDNNVGLALGPPQSSSFQTMTSSLPQTNLNPFEDWPHNRD 444
ZmCBP     QTPYDYTFSFLPPNLLTGSAND-AMGLELG--QLHQVISQGQPIQPANVG-YVDWPRNRD 512
WCBP1     ----------VNTHYFQ----------------GESILPVGQQQPTIFTGELLHWPYER- 484
                     .: :                   .. .  .       .   .** :*

NtCBP60   KGVDEFLSEEEIRMRSHEILENDDMQHLLRLFSMGGGHGSVNVSEDGYGFPSFMPSPSPT 504
ZmCBP     SQYADDFT-EDIRVKSHQMLESEDMQQLLRVFSMGGASG--SLSDDTFNFQPYMPSPLPN 569
WCBP1     ---------------SGQVNIHSQMQAPMSMDGGGVIQASTSVQHN-LLPQLFVPTQVQE 528
                        * ::   .:**  : : . *   .  .:..:      ::*:

NtCBP60   FGYD-EDPKPSGKAVVGWLKIKAAMRWGFFVRKKAAERRAQIVELDDE 551
ZmCBP     PGFESERSHSSGKAVVGWLKIKAAMRWGIFVRKKAAERRAQLVELED- 616
WCBP1     SMHAIHYSENPPNHAVGPSTWHDDIYP---------------------- 555
            .  . .. . : .**  . :  :
```

◀ **Fig. 5** Alignment of the predicted amino acid sequence of WCBP1 with NtCBP60 of tobacco and ZmCBP of maize. The sequences for NtCBP60 and ZmCBP were obtained from the NCBI database (see Table 2) and aligned using clustalw2. Identical and similar amino acid residues in all sequences are indicated by *asterisks* and *dots*, respectively. *Hyphens* indicate gaps introduced to facilitate alignment. Amino acid residues indicated by *red lines* form the consensus amino acid region of the CaM-binding domain, which is detected in the CBP60 family such as NtCBP60 and ZmCBP. Amino acid residues indicated by *blue lines* form domains specific to CaM-binding proteins and were identified by the domain analysis using ProDom program

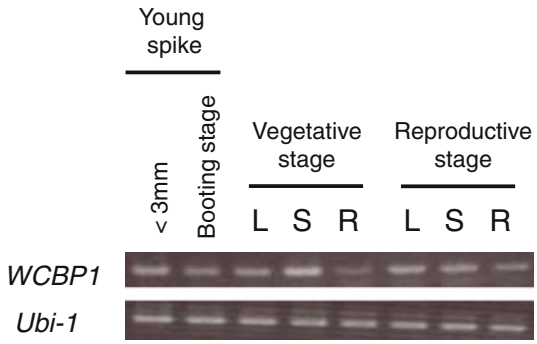
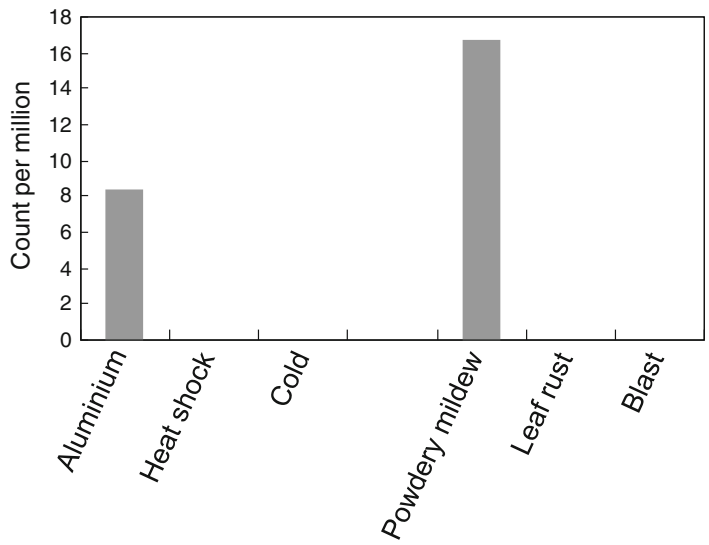


Fig. 6 RT-PCR expression patterns of *WCBP1* in young spikes (<3 mm in length and at the booting stage), in seedling leaves (*L*), stems (*S*) and roots (*R*) at the vegetative (3-leaf) stage, and in plants at the reproductive (floret differentiation) stage. The *Ubiquitin* (*Ubi-1*) gene was used as the control, to ensure that the same amount of cDNA was used in the PCR for each tissue. PCR patterns in the exponential amplification phase are shown

accumulated throughout the whole floral organ including the pistil and stamens (Fig. 8e, g). The level of expression decreased at the late stage of floral organ formation (Fig. 8i). *WCBP1* mRNA was detected across a wider region of the spikelet primordia of young florets of a (cr)-CSdt7BS plant compared to a plant of the normal line (Fig. 8b). *WCBP1* mRNA accumulated in the primordia of the pistil and the pistil-like stamens (Fig. 8d). Expression continued at the floret differentiation stage when the pistil and pistilloid stamens develop (Fig. 8f, h). By comparison to control plants, *WCBP1* mRNA showed greater accumulation and expression at a higher level in the late stage of floral organ formation in (cr)-CSdt7BS plants (Fig. 8j).

Overall, our observations suggest that *WCBP1* expression is more widely distributed at early stages of floret development and also shows increased expression that is associated with the formation and development of pistil-like stamens as well as pistils in alloplasmic (cr)-CSdt7BS plants.

Fig. 7 In silico expression pattern of *WCBP1* in seedlings subjected to abiotic stress (aluminum, cold or heat shock) or to biotic stress (powdery mildew, leaf rust or blast infection). The names of the cDNA libraries, the numbers of total ESTs, and the numbers of *WCBP1* ESTs are shown. Expression levels are represented as the ratio of *WCBP1* ESTs to each total EST (count per million)



	Abiotic stress			Biotic stress		
Library name	SCTAL	VHS	V4816	CHUL	XF	XP
No. of Total EST	24171	17735	18903	24171	18949	22208
No. of *WCBP1* EST	2	0	0	4	0	0

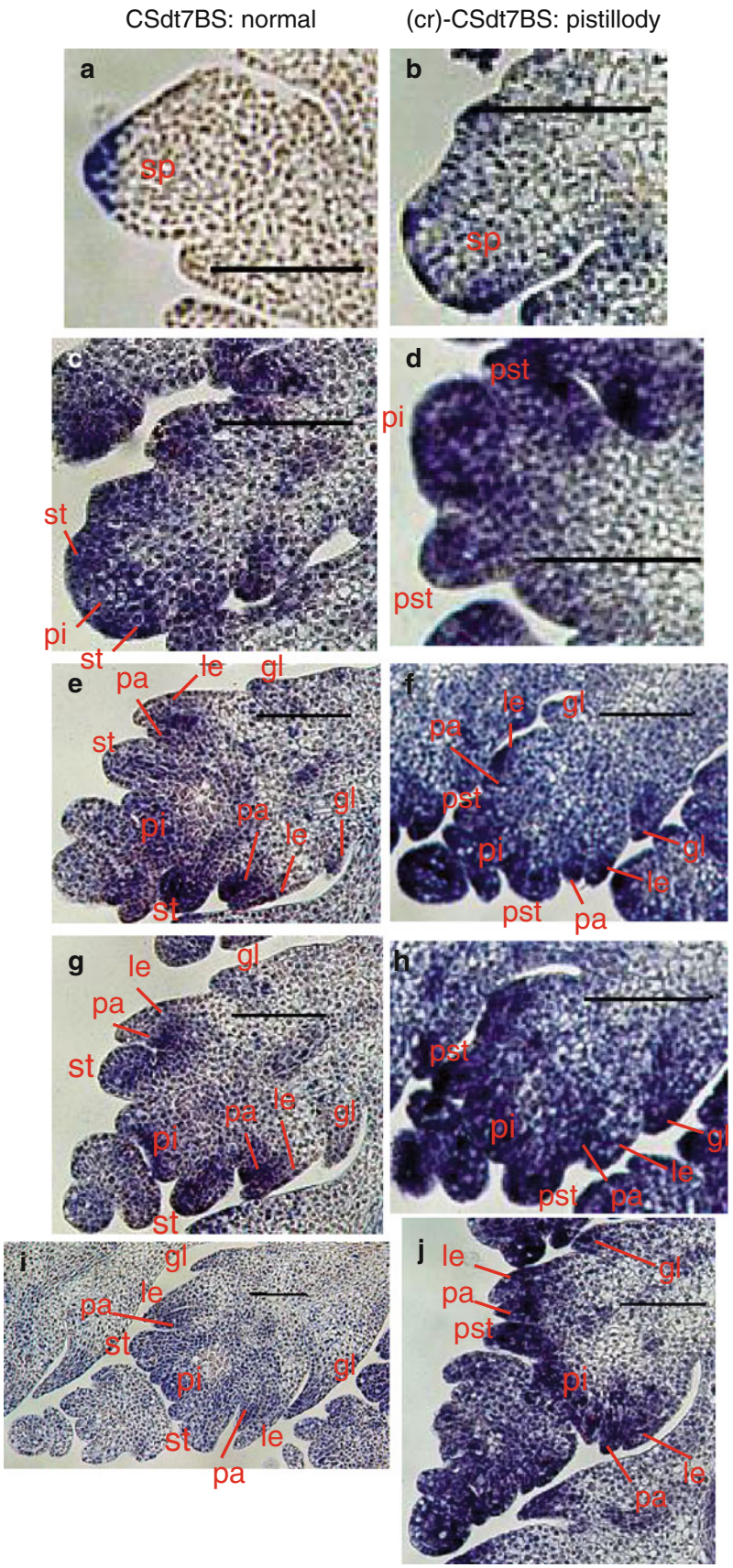


Fig. 8 In situ hybridization analysis of *WCBP1* transcripts in spikelets from the normal CSdt7BS line (**a**, **c**, **e**, **g**, **i**) and the pistillody (cr)-CSdt7BS line (**b**, **d**, **f**, **h**, **j**). **a**, **b** Spikelet primordia. **c**, **d** Early floret differentiation stage. The spikelet from the normal plant is at a slightly earlier stage of development than that of the pistillody plant in which primordia of the pistil and stamens have just initiated. **e**, **f** Floret differentiation stage. The spikelet of the normal plant is at a slightly later stage than that of the pistillody line. **g**, **h** Late floret differentiation stage. **i**, **j** Floral organ developing stage. *pi* pistil, *st* stamen, *pst* pistilloid stamen, *pa* palea, *le* lemma, *gl* glume; *bars* 0.05 mm

Discussion

Cytoplasmic male sterility (CMS) is the best-known phenomenon caused by MRS in plants (Fujii and Toriyama 2008). CMS is characterized by failure to produce viable pollen and is the most frequent effect of an alien cytoplasm (mitochondrial genome), and usually occurs following recurrent backcrossing in which a related wild species is used as the first female parent and the elite cultivar is used as the recurrent male parent (Kaul 1988). In crop species, CMS is of value for hybrid seed production: the CMS line is unable to self-pollinate, and pollination is therefore dependent on pollen from the male line. In many plant species, CMS is associated with chimeric or novel mitochondrial open reading frames (ORFs) (Hanson and Bentolila 2004; Chase 2007). These unique ORFs are transcribed and translated into proteins that appear to interfere with mitochondrial function and pollen development. In many cases, nuclear restorer (*Rf*) genes suppress the male sterility induced by CMS-associated mitochondrial genes. In several plant species such as petunia, *Brassica* and rice, *Rf* genes encode pentatricopeptide repeat proteins, which are thought to be RNA binding proteins involved in posttranscriptional processing of mitochondrial genes (Hanson and Bentolila 2004; Chase 2007).

Pistillody (Fig. 1) is a type of CMS in wheat (Murai et al. 2002). We previously reported that *orf260* is the mitochondrial factor and *Rfd1* is the fertility restoring gene in the nuclear genome (Murai and Tsunewaki 1994; Zhu et al. 2008). Furthermore, we demonstrated that pistillody induction is caused by an alteration to class B MADS-box gene expression in stamen primordia at floral whorl 3 (Hama et al. 2004). Cytoplasmically induced pistillody is also known in tobacco (*Nicotiana tabacum*) (Kofer et al. 1991; Zubko et al. 1996), carrot (*Daucus carota*) (Linke et al. 1999), and *Brassica napus* (Leino et al. 2003), where it is associated with downregulation of class B genes (Zubko et al. 2001; Linke et al. 2003; Teixeira et al. 2005). These observations together with the present results indicate that a common mechanism is involved in the homeotic transformation of stamens into pistil-like structures in alloplasmic plants. This phenomenon has been named 'cytoplasmic homeosis' (Zubko 2004).

Our microarray analysis identified five candidate genes (contigs) for the cytoplasmic homeosis displayed by plants of the alloplasmic (cr)-CSdt7BS line (Table 1). RelA/SpoT protein (Ctg6837) is involved in the calcium-dependent production of a small signaling nucleotide, guanosine 5′-diphosphate 3′-diphosphate, that is found mainly in the chloroplast, indicating its relationship with chloroplast signaling (Stael et al. 2012). F-box proteins (Ctg9545) are encoded by a large gene family and regulate diverse cellular processes, including transcriptional regulation and signal transduction (Kuroda et al. 2002). In *Arabidopsis*, F-box proteins such as UNUSUAL FLORAL ORGANS (UFO) act as a transcriptional co-factor to regulate floral homeotic genes (Chae et al. 2008). However, real-time PCR analysis did not confirm the significant upregulation of either Ctg6837 or Ctg9545 (Fig. 2). CRAL/TRIO protein (Ctg2065) is likely to bind a small hydrophobic ligand and is a critical modulator of cell gate specification during *C. elegans* development (Johnson and Kornfeld 2010). We also did not confirm the upregulation of Ctg2065 in the pistillody line by real-time PCR analysis (Fig. 2).

In our previous study, we identified a protein kinase gene, *WPPK1* (*wheat pistillody-related protein kinase 1*), which is upregulated in the young spikes of the pistillody line (Saraike et al. 2007). The amino acid sequence of WPPK1 shows high similarity to a flowering plant PVPK-1 protein kinase, and a phylogenetic analysis indicated that WPPK1 is a member of the AGC group protein kinases. In situ hybridization analysis revealed that *WPPK1* is expressed in developing pistils and pistil-like stamens as well as in their primordia. These findings indicate that in the alloplasmic line, *WPPK1* plays a role in the formation and development of pistil-like stamens. However, the protein kinase gene (Ctg10001) identified in this study was not identical to *WPPK1* (data not shown). We confirmed the upregulation of Ctg10001 in the pistillody line by real-time PCR analysis, but its function with respect to pistillody induction is unclear.

Real-time PCR analysis and in situ expression analysis confirmed that a CaM-binding protein gene (Ctg4015), named *WCBP1* in this study, was significantly upregulated in the pistillody line compared with normal plants (Fig. 2). *WCBP1* is a novel member of the CaM-binding protein gene family and was expressed in young spikes at the floret differentiation stage (Figs. 3, 4, 5). CaM is a widely distributed family of proteins in eukaryotes including plant species. The proteins act as a primary intracellular Ca^{2+} receptor in all eukaryotes (Zielinski 1998). Ca^{2+} is a second messenger in relaying developmental and environmental signals to the appropriate cellular locations in plants and animals (Ranty et al. 2006; Kim et al. 2009). In mammalian cells, mitochondria play an important role in Ca^{2+} storage and Ca^{2+} homeostasis (Szabadkai and Duchen 2008). Therefore, Ca^{2+} signaling is likely to be a one of the main factors in MRS (Butow and Avadhani 2004). Although *WCBP1* probably functions in various tissues in a range of developmental stages as well as in biotic/abiotic stress signaling pathways (Figs. 6, 7), the present study indicates that the CaM-binding protein, *WCBP1*, was associated in pistillody induction caused by MRS in wheat. The findings in the present investigation support the idea that Ca^{2+} signaling is an important factor in MRS in plants.

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